

INDUCTION OF APOPTOSIS IN MCF-7 CELLS BY METHANOLIC EXTRACT OF *CLITORIA*
TERNATEA L.

¹Priyanka Shivaprakash, ²Kyathgowdanadoddi Srinivasa Balaji, ³Kagepura Thammaiah Chandrashekara,
⁴Kanchugarakoppal Subbegowda Rangappa, ⁵Shankar Jayarama*.

^{1,2}Junior Research Fellow, P.G. Department of Biotechnology, Teresian College (Affiliated to University of Mysore), Siddhartha Nagar, Mysore-570011, India.

³Institution of Excellence, University of Mysore, Manasagangotri, Mysore-570006, India.

⁴Department of Studies in Chemistry, University of Mysore, Manasagangotri, Mysore-570006, India.

⁵P.G. Department of Biotechnology, Teresian College (Affiliated to University of Mysore), Siddhartha Nagar, Mysore-570011, India and Department of Microbiology & Immunology, University of Illinois at Chicago, Chicago-60612, USA.

*Corresponding Author E-mail: shankarbio@gmail.com, Telephone: 0821-2471316, Fax: 0821-2476997

ABSTRACT

Aim: Investigation of apoptosis induction by methanolic extract of *Clitoria ternatea* L. flower against multiple cancer cell lines.

Main Methods: In the present study cytotoxic activity of *Clitoria ternatea* L flower was determined using MTT cell viability assay. The induction of cell death/apoptosis was evaluated by light microscopy, DNA fragmentation and caspase-3 enzyme activation.

Key Findings: The methanolic extract from *C. ternatea* (MECT) showed cytotoxic activity against several cancer cell lines. The most potent activity exhibited by MECT was against MCF-7 breast carcinoma cells with an IC₅₀ value of 27.2 ± 2.6 µg/mL. Light microscopic evaluation clearly indicated the apoptotic morphology of MECT treated cells. Treatment of MCF-7 cells with various MECT concentrations resulted in growth inhibition and induction of apoptosis as indicated by DNA fragmentation and caspase-3 enzyme activation.

Significance: The current report strongly suggests the pro-apoptotic properties of *C. ternatea* flowers. Our findings demonstrate that *C. ternatea* phyto-constituents may have beneficial applications in the field of anti-cancer drug discovery.

Keywords: *Clitoria ternatea*, Apoptosis, MTT assay, DNA fragmentation, Caspase-3.

INTRODUCTION

The distinguishable feature of cancer cell is its ability to circumvent apoptosis or programmed cell death, a highly regulated cellular process which maintains tissue homeostasis through balance between cell proliferation and cell death (Fulda, 2010). Apoptosis acts like suicide programme by which cells dies without harming adjacent cells as the apoptotic bodies are readily phagocytosed by macrophages without triggering immune response (Indran et al., 2011). The cells that are undergoing apoptosis exhibit distinct morphological characteristics such as chromatin condensation, membrane blebbing, reduction in cellular volume (pyknosis), retraction of pseudopodes and nuclear fragmentation are similar in all cells with irrespective of their cell type and physiological or pathological conditions (Wong, 2011). The defective apoptotic machinery due to genetic mutations or epigenetic modifications leads to enhancement of tumor cell proliferation and is responsible for establishment of metastasis (Su et al., 2015).

As a result of decades of research towards finding effective natural agents to combat against cancer, good number of more than 3000 plants has been reported with anti-cancer property (Millimouno et al., 2014). Beta-lapachone (Li et al., 1995), genistein (Banerjee et al., 2008), resveratrol, lycopene, epigallocatechin-3-gallate (EGCG) (Russo et al., 2008), lupeol, fisetin (Khan et al., 2010), apigenin (Wang et al., 2012) are some of the plant derived compounds which have successfully displayed their potentiality in inducing cancer cell apoptosis. *Clitoria ternatea* is a well-known medicinal plant in ayurveda, commonly known as 'butterfly pea' belongs to the family fabaceae (Zingare et al., 2013). Plant possess variety of phytochemicals which have been reported to be malonylated flavonoid glucosides; kaempferol, quercetin and myricetin (Gupta et al., 2010; Kazuma et al., 2003), teranthocyanins, anthocyanins; ternatins (Terahara et al., 1998; Terahara & Oda, 1996), terpenoids; taraxerol and taraxerone, steroids; β -sitosterol, γ -sitosterol (Bisby, 1994). In ayurveda the plant is extensively used to enhance memory and intelligence. The different parts of plant possess nootropic, antistress, anxiolytic, antimicrobial, diuretic, insecticidal, blood platelet aggregation-inhibiting properties (Mukherjee et al., 2007, Kamkaen & Wilkinson, 2009). Plant also shows hepatoprotective activity (Nithianatham et al., 2013), antitumor activity (Jacob & Latha, 2012), anti-inflammatory, antidiabetic (Suganya et al., 2014; Daisy & Rajathi, 2009), anti-glycation activity (Chayaratanasin et al., 2015). *C. ternatea* proved to have chemosensitization in paclitaxel resistant lung cancer cell line (Sen et al., 2013). In another study water extract from flowers of *C. ternatea* exhibit cytotoxic effect on various cell lines such as MCF-7, MDA-MB-231, Caov-3, HeLa, HepG2 and Hs27 (Neda et al., 2013).

Based on the reports we selected *C. ternatea* to investigate its anti-cancer effect on various cancer cell lines and herein we have reported the preliminary work and the possible mechanism of *C. ternatea* inducing tumor cell apoptosis

METHODS

Plant material

The flowers of *Clitoria ternatea* L. were collected from Western Ghats, India in March 2013. Dr. S. Lokesh of Biotechnology Department, University of Mysore, Mysore, India botanically authenticated the plant. A voucher specimen number TC-09/2013 describing the plant was deposited at the University.

Extract preparation

The extracts were prepared according to World Health Organization protocol (CG-1983) with a slight modification. The flowers were dried and powdered, 25 gm powder was extracted with 300 mL of distilled water, methanol or ethanol (3 times) for 3 h under reflux. After extractions, the extracts were combined and filtered with Whatman filter paper No.1, and then were concentrated in vacuum to dryness. All extracts were stored at -4°C until use.

Cell lines and culture

SK-BR-3 (Human breast carcinoma), MCF-7 (Human breast adenocarcinoma), Hep3b (Human hepatocellular carcinoma), K562 (Human leukemia cells), 8505C (Human anaplastic thyroid cancer), cells were provided by Dr. B.S. Prabhakar, Department of Microbiology & Immunology, University of Illinois at Chicago, Chicago, USA. The SK-BR-3 cells were maintained in McCoy's 5A, MCF-7 and Hep3b in MEM, K562 in IMDM and 8505C in RPMI 1640 (Gibco-Invitrogen, NY, USA) with 10% fetal bovine serum (FBS) supplemented with 2% penicillin and 100 $\mu\text{g/mL}$ of streptomycin at 37°C in a humidified atmosphere containing 5% CO_2 .

Cell viability assay

Direct interference between different concentrations of MECT (5–100 $\mu\text{g/mL}$) and MTT in a cell-free system was not observed, therefore MTT assay was used to test cell viability in the current system. SK-BR-3, MCF-7, Hep3b, K562 and 8505C cells ($4-6 \times 10^3/\text{well}$) were cultured in 96-well plates and after 24 h the cells were treated with different concentrations of MECT (5–100 $\mu\text{g/mL}$), curcumin (5 $\mu\text{g/mL}$) for 24 h at 37°C . Cell viability was assessed by MTT (0.5 mg/mL) conversion as described previously (Lasek et al., 1995).

Agarose gel electrophoresis (DNA laddering)

MCF-7 cells (5×10^5 cell/mL) were treated with the indicated concentration of test extract for different time period. After the supernatant was removed by centrifugation (1500 rpm, 4°C), the cells were washed with 1 mL of PBS and was precipitated by centrifugation at 3000 rpm for 10 min at 4°C . Cells were lysed in a lysis buffer containing 50 mM Tris-HCl, pH 8.0 and 0.5% SDS and incubated for 30 min at 37°C . The cell lysate was subjected to 8 M potassium acetate precipitation and left for 1 h at 4°C . The supernatant was subjected to phenol/chloroform/isoamyl alcohol (25:24:1) extraction followed by chloroform extraction. DNA was precipitated by adding 1:2 volumes of ice-cold ethanol. The precipitated DNA was digested with 20 $\mu\text{g/mL}$ RNase at 37°C for 30 min. The DNA (1 μg) was resolved on 1.2% agarose gel in TAE buffer and documented using UVP-Bio Doc It TM system.

Caspase-3 activity

Caspase-3 enzyme activity was measured by proteolytic cleavage of the fluorogenic substrate Ac-DEVD-AFC by counting on a fluorescence plate reader as described previously (Hung et al., 2014). MCF-7 cells (1×10^5 cell/well) were treated with plant extract at concentrations of 6.25, 12.5, 25, 50 and 100 $\mu\text{g/mL}$. After incubation for different time period, cells were harvested and washed with cold PBS. The pellets were lysed using 15 μL of lysis buffer [10 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.5% Triton X-100] at room temperature for 10 min, and then placed on ice; 100 μL of assay buffer [100 mM Hepes (pH 7.5), 10 mM dithiothreitol, 10% (w/v) sucrose, 0.1% (v/v) Chaps, 0.1% (v/v) BSA] and 10 μL of substrate solution (200 μM substrate in assay buffer) were added. After incubation at 37 °C for 1 h, fluorescence was measured with excitation at 370 nm and emission at 505 nm.

Statistical analysis

The results are expressed as mean value \pm S.D. Statistical analysis was performed using one-way ANOVA. A P <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Induction of apoptosis selectively in cancer cells is one of the prime aim of cancer therapy. Recently medicinal plants have become the source of alternative therapy for cancer treatment due to their safety and efficacy where they simultaneously influence different phases of diseases through different mechanisms. Almost 90 out of 121 drugs prescribed to treat cancer are originated from plants (Safarzadeh et al., 2014). *C. ternatea* is a plant with many medicinal values which is been used for many centuries in ayurvedic system of medicine in India.

The aqueous, methanol and ethanol extract yields from *C. ternatea* flowers were 20.2, 22.4, and 8.5% respectively (Table 1). The inhibitory effect on cancer cell proliferation and subsequent induction of cell death by MECT was demonstrated on various human cancer cell lines such as HeLa, MCF-7, SK-BR-3, K562 and 8505C. The cytotoxic effect of MECT on multiple cell line was assessed by performing MTT assay. Different concentrations of plant extract (6.25, 12.5, 25 and 50 $\mu\text{g/mL}$) were used to treat the cells along with standard anticancer drug curcumin (5 to 10 $\mu\text{g/mL}$). The cytotoxic effect of MECT was found to be more on MCF-7 with IC_{50} value of 27.2 ± 2.6 $\mu\text{g/mL}$, when compared to SK-BR-3, Hep3b, K-562 and 8505C with IC_{50} value of 47.2 ± 2.6 $\mu\text{g/mL}$, 72.5 ± 3.6 $\mu\text{g/mL}$, 48.5 ± 1.8 and >100 $\mu\text{g/mL}$ respectively (Table 2). Further the anti-tumor effect of MECT was evaluated by treating MCF-7 cells with plant extract for 48 h. Total numbers of viable cells were counted using trypan blue exclusion method. Effective reduction in cell viability was observed at the concentration of 25 $\mu\text{g/mL}$ of MECT with reduced cell number of 12×10^4 cells/mL compared to untreated cells which are having viability of 29×10^4 cells/mL.

Table-1. Percentage yields of extracts (% w/w)

Plant extracts	Yield (%)
Aqueous Extract	20.2
MeOH Extract	22.4
EtOH Extract	8.5

Table-2. Cytotoxic activity of extracts from *C. ternatea*

Extracts	IC_{50} value ($\mu\text{g/mL}$) ^a				
	SK-BR-3	MCF-7	Hep3b	K562	8505C
Aqueous Extract	51.4 ± 2.8	38.4 ± 2.1	>100	68.2 ± 2.7	>100
MeOH Extract	47.2 ± 2.6	27.2 ± 2.6	72.5 ± 3.6	48.5 ± 1.8	>100
EtOH Extract	55.8 ± 4.2	42.8 ± 3.6	>100	>100	>100
Curcumin ^b	15.5 ± 1.8	8.5 ± 1.2	18.9 ± 2.6	16.2 ± 0.8	25.4 ± 1.3

^aThe inhibitory effects are represented as giving 50% inhibition (IC_{50}) relative to the vehicle control. These data represent the average values of three repeated experiments (mean \pm S.D.). ^bPositive control.

The cells dying due to apoptosis display distinct morphological features with condensed cytoplasm and nucleus, aggregated chromatin and formation of membrane-bound vesicles known as apoptotic bodies (Fig 1). To verify whether the ability of MECT induced cell death is due to apoptosis the pattern of DNA bands on agarose gel were analyzed. Chromosomal DNA extracted from cells treated with 25 or 50 $\mu\text{g/mL}$ MECT for 24 h exhibited inter nucleosomal DNA fragmentation which is a hallmark of cells undergoing apoptosis (Fig 2).

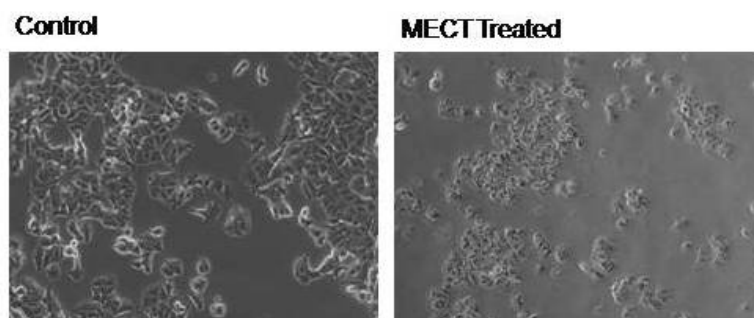


Figure 1. Light microscopic evaluation of MCF-7 cells. MCF-7 cells were treated with or without MECT and the morphology of cell was captured and documented.

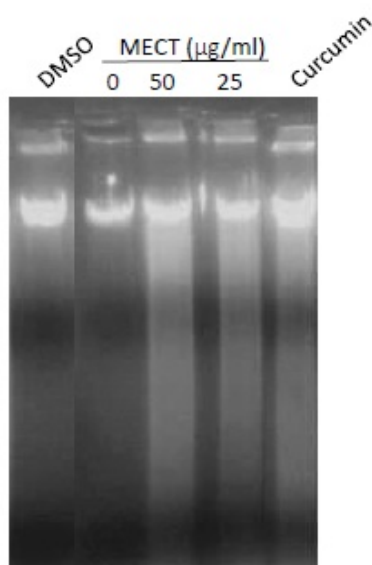


Figure 2. Induction of DNA fragmentation in MCF-7 cells *in vitro*. MCF-7 cells were treated with MECT for 24 h at 50 $\mu\text{g/mL}$ and 25 $\mu\text{g/mL}$. Total genomic DNA was extracted and resolved on 1.2% agarose gel. Apoptotic DNA fragmentation was visualized by ethidium bromide staining.

As apoptosis is mediated by caspases, the activity of caspases-3 an executor of DNA fragmentation and apoptosis was measured. Normally the caspases are present in dormant state and are activated upon apoptotic stimulation. Activity of Caspases-3 was measured by the amount of proteolytic cleavage of substrate Ac-Asp-Glu-Val-Asp-8-amino-4-trifluoromethylcoumarin (Av-DEVD-AFC). Increased activity of caspase-3 was observed in MCF-7 cells upon treatment with MECT (25 $\mu\text{g/mL}$) after 48 h (Fig 3). The anti-cancer activity of medicinal plants is mainly attributed to polyphenols including flavonoids which also exhibit strong anti-oxidant activity (Hu, 2011). The group of flavonoids consist more than 4000 varieties which makes 60% of total polyphenols found naturally in plants (Chaharet al., 2011). Quercetin, myricetin, kaempferol are the members of flavonol which are found in many vegetables and fruits have been proven to be effective inhibitors of human prostate, lung, stomach, colon, leukemia, thyroid, breast cancer cell growth (Kanadaswami et al., 2005; Batra & Sharma, 2013). Studies have demonstrated that taraxerol, a triterpenoid exhibit anti-cancer effect on sarcoma 180 cell line, spontaneous mammary tumors in mice (Sharma & Zafar, 2015). Beta-sitosterol, a major phytosterol has also been shown to induce apoptosis in many cancer cell lines (Park et al., 2007; Awad et al., 2000a & 2000b).

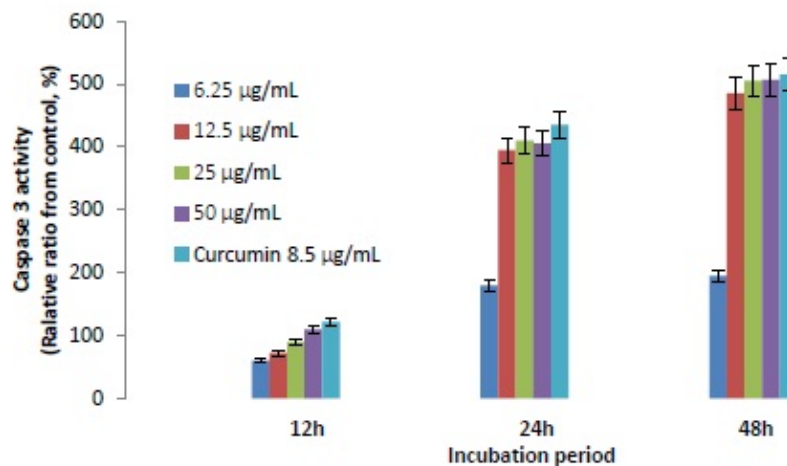


Figure 3. Caspase-3 activity in MCF-7 cells after *in vitro* treatment: MCF-7 cells were treated with different concentrations of MECT for 12, 24 and 48 h, cell lysates were prepared and incubated with caspase-3 substrate (Ac-DEVD-AFC) for 1 h at 37 °C. The fluorescence intensity of the cell lysate was measured to determine the caspase-3 activity. The blank group was used as 0.1% DMSO-treated cells; Curcumin (8.5 µg/mL) was used as positive control. Data are presented as the mean \pm S.D. of results from three independent experiments (* $P < 0.05$ vs. Control).

This particular study demonstrated the effect of MECT in inducing cancer cell apoptosis. Currently out of five cell lines tested the maximum anticancer effect of MECT was exerted on MCF-7. As a proof significant reduction of cell viability was observed this was confirmed by MTT assay followed by cell count using trypan blue exclusion method. In addition DNA gel electrophoresis revealed the ladder pattern which is a key feature of apoptosis. Further increased activity of caspase-3 activity upon treatment was a strong evidence to show the MECT as better anticancer phytoresource which can induce apoptosis in cancer cells.

CONCLUSION

The preliminary report of current investigation clearly evident that MECT effectively inhibit the proliferation of MCF-7 cells by the mechanism which involves the induction of apoptosis. According to these results, it is suggested that the MECT may be a considerable source for the development of anti-cancer drug. Currently we are investigating the bioactive components which are responsible for induction of apoptosis.

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Conflict of Interest: The authors declare there is no conflict of interests.

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